Does multiple paternity affects seed mass in angiosperms? An experimental test in Dalechampia scandens

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Abstract

Flowers fertilized by multiple fathers may be expected to produce heavier seeds than those fertilized by a single father. However, the adaptive mechanisms leading to such differences remain unclear, and the evidence inconsistent. Here, we first review the different hypotheses predicting an increase in seed mass when multiple paternity occurs. We show that distinguishing between these hypotheses requires information about average seed mass, but also about within fruit variance in seed mass, bias in siring success among pollen donors, and whether siring success and seed mass are correlated. We then report the results of an experiment on Dalechampia scandens (Euphorbiaceae), assessing these critical variables in conjunction with a comparison of seed mass resulting from crosses with single vs. multiple pollen donors. Siring success differed among males when competing for fertilization, but average seed mass was not affected by the number of fathers. Furthermore, paternal identity explained only 3.8% of the variance in seed mass, and siring success was not correlated with the mass of the seeds produced. Finally, within infructescence variance in seed mass was not affected by the number of fathers. These results suggest that neither differential allocation nor sibling rivalry has any effect on the average mass of seeds in multiply sired fruits in D. scandens. Overall, the limited paternal effects observed in most studies and the possibility of diversification bet hedging among flowers (but not within flowers), suggest that multiple paternity within fruits or infructescence is unlikely to affect seed mass in a large number of angiosperm species.

Key words: parent offspring conflict, sexual conflict, differential allocation, seed size, pollen competition, paternity analysis, diversification bet hedging, Dalechampia

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Introduction

In angiosperms, cross pollination often results in multiple paternity in multi seeded fruits (Ellstrand, 1984; Ellstrand & Marshall, 1986; Snow, 1994; Ibarra Perez et al., 1996; Bernasconi, 2004; Teixeira & Bernasconi, 2007; Pannell & Labouche, 2013). The various plant features that increase the potential for multiple paternity, such as functional syncarpy (Endress 1982; Armbruster et al., 2002), enlarged stigmatic surface (Armbruster, 1996), and delayed stigma receptivity (Galen et al., 1986; Lankinen et al., 2007; Lankinen & Madjidian, 2011), further suggest that plants may benefit from having the seeds within each fruit or infructescence sired by multiple pollen donors (Pannell & Labouche, 2013). This hypothesis is also supported by the selective maturation of multiply sired over singly sired fruits observed in Raphanus sativus (Marshall, 1988). If seeds from multiply sired fruits have higher reproductive potential, due to either the benefits of genetically diverse offspring filling alternative niches or the selective process of pollen competition resulting in genetically better quality offspring, then maternal plants may adaptively allocate more resources to those seeds in order to increase their probability of recruitment (Temme, 1986). Alternatively, the decrease in genetic relatedness among seeds generated by multiple paternity may increase the level of sibling competition for maternal resources and, in turn, create an apparently preferential bias of seed provisioning within the same fruit by maternal plants (Kress, 1981; Mock & Parker, 1997).

Over the past four decades, several authors have adopted one or more of these alternative hypotheses and predicted that average seed mass should be affected positively by the occurrence of multiple paternity within fruits (Kress, 1981; Westoby & Rice, 1982; Queller, 1983; Mazer, 1987; Shaanker et al., 1988; H‰rdling & Nilsson, 1999). However, studies that compare seed mass obtained from fruits pollinated by single vs. multiple pollen donors report inconsistent patterns. Out of the 11 studies that explicitly tested the effects of

multiple paternity on seed mass, only two found evidence for seed mass increasing with the number of pollen donors (Schemske & Pautler, 1984; BaÒuelos & Obeso, 2003; Table 1). Furthermore, in the study by Schemske and Pautler (1984), the increase in the number of pollen donors was associated with an increase in genetic diversity of pollen donors and receivers, thus potentially confounding the effects of inbreeding depression and the number of pollen donors (Mazer, 1987; Charlesworth, 1988). Additionally, in several cases, the use of a limited number of individuals acting either as pollen donors or receivers limited the generality of the study, because specific individual interactions could have produced the observed results (e.g. Bertin, 1986; Marshall, 1988; Snow, 1990).

These considerations underline the large variety of mechanisms that may influence seed mass when the number of pollen donors varies. Here, we first review the different hypotheses that have been put forward to predict an increase in seed mass with multiple paternity in angiosperms. We show how one can distinguish between these different hypotheses by observing not only the change in mean seed mass, but also the within fruit (or within infructescence) variance in seed mass, the existence of paternal genetic effects on seed mass, and whether or not paternal plants differ in their siring abilities. We then present results from a crossing experiment on Dalechampia scandens (Euphorbiaceae), in which these critical variables have been measured.

Effects of multiple paternity on average seed mass: reviewing the hypotheses

We group the various hypotheses predicting an increase in average seed mass with multiple paternity into four categories, briefly referred to as: (i) genetic diversity, (ii) differential allocation, (iii) sibling rivalry and (iv) parent-offspring conflict and relatedness (Table 2). All hypotheses assume that an increase in seed mass will positively affect one or more fitness components of the offspring, such as germination success (Stanton, 1984; Paz et al., 1999;

93 Dalling & Hubbell, 2002), early seedling growth (Stanton 1984; Vaughton & Ramsey, 1998;
94 Bretagnolle et al., 1995; PÈlabon et al., 2005), or survival and recruitment (Winn, 1988;
95 Tremayne & Richards, 2000; Halpern, 2005). However, the mechanisms generating the
96 increase in seed mass differ among hypotheses. These include differential allocation of
97 resources by the maternal plant (Temme, 1986) or variation among half sibs in their ability to
98 convert (Temme, 1986) or extract (House et al., 2010; Ulloa & Mera, 2010) maternal
99 resources. Operation of these mechanisms has been discussed in the literature (e.g. genetic
100 imprinting for genes affecting seed size: Haig & Westoby, 1991; de Jong and Scott, 2007;
101 epigenetic control of resource accumulation by maternal and paternal alleles: Li & Dickinson,
102 2010), and we simply assume here that these mechanisms can occur. We also note that
103 variation among half sibs in their ability to convert or extract maternal resources will often be
104 expressed as paternal (additive genetic) effects on seed mass (Mazer & Gorchov, 1996).
105
106 Maternal investment in genetically diverse broods ñ In this first group of hypotheses, it is
107 assumed that the increased genetic diversity of seeds sired by genetically different pollen
108 donors increases the reproductive potential of the whole brood. Here, we use the term
109 ìbroodî to refer to the seeds produced by a single fruit or infructescence. (See Methods for a
110 discussion on the possible difference of effects between fruit and infructescence.) Maternal
111 plants that are able to allocate more resources to these broods will increase their fitness by
112 doing so, because it will increase the probability of members of the brood as a whole
113 reaching maturity and reproducing. Two mechanisms have been suggested to explain the
114 increase in reproductive potential of genetically diverse broods (Karron & Marshall, 1993).
115 First, in unpredictable, temporally or spatially variable environments, genetic diversity of the
116 brood is expected to increase the probability that some individuals are well adapted to the
117 environment encountered. This hypothesis, referred to as the ìlottery hypothesisî by Karron

and Marshall (1993) is perhaps more generally known as diversification bet hedging (Watson 1991; Yasui, 1998; Fox & Rauter, 2003; Starrfelt & Kokko, 2012), and involves here genetic as well as just phenotypic diversity in offspring. Alternatively, in constant environments, competition after dispersal may be less intense among half sibs than among full sibs, due to more effective resource partitioning (the ìelbow room hypothesisî in Karron & Marshall, 1993; see also Bulmer, 1980; Barton & Post, 1986; McLeod & Marshall, 2009; Aguirre & Marshall, 2012; and File et al., 2012 and Dudley et al., 2013 for recent reviews).

In both scenarios, the whole brood has a higher reproductive potential when sired by multiple pollen donors. Therefore, differential maternal allocation should be directed equally towards every seed in genetically diverse broods, and we do not expect an increase in the within brood variance in seed mass (at least after scaling for the increase in mass). Additionally, neither paternal effects on seed mass nor biased paternity (different siring ability among paternal plants) should be associated with this increase in seed mass, since the maternal plant is the one promoting diversity within the brood (McLeod & Marshall, 2009). Consequently, under the genetic diversity hypothesis, we expect multiply sired fruits to contain seeds of a higher average mass, but have no fewer seeds per fruit, show no increase in the variance in seed mass, and exhibit unbiased paternity. Although paternal effects on seed mass may be observed, their occurrence is not required for the increase in average seed mass under these hypotheses.

Differential allocation ñ If the ability to win pollen competition is correlated with the genetic quality of the seed produced (e.g. Marshall 1991; Skogsmyr & Lankinen 2002; but see Lankinen et al. 2009), maternal fitness is expected to increase if the maternal plant allocates more resources towards these genetically superior seeds in order to increase their recruitment probability. Additionally, if multiple paternity within fruit is associated with the occurrence

of pollen competition, we expect a positive correlation between multiple paternity and average seed mass (Marshall & Ellstrand, 1986). This requires that either embryo maternal plant interactions allow the latter to distinguish among embryos and to differentially allocate resources to those with the highest quality, or that a genetic correlation exits between siring ability and the embryoís ability to convert or extract maternal resources during seed maturation, thereby allowing high quality seeds to receive extra investment.

These various scenarios predict that polyandrous mating (i.e. pollination by several pollen donors) should generate biased paternity in favor of certain male plants that will sire larger seeds. Therefore, multiple sired broods with biased paternity should be heavier than single sired broods only on average. This is because occasional broods sired entirely by one pollen donor of high quality should be just as heavy, if not heavier, than multiple sired broods. Although an increase in within brood variance in seed mass is expected in broods with multiple fathers compared to broods with only one father, selection via pollen competition for offspring with bigger seed mass will possibly limit this increase, once scaled for the mass differences.

These scenarios strongly depend upon the existence of a correlation between the sire success in pollen competition and the quality of the seed produced in order to generate the increase in seed mass in multiply sired broods. However, one can also imagine that the quality of the embryo depends upon non additive genetic effects (i.e. gene compatibility, Neff & Pitcher, 2005), and that maternal plants select among pollen grains based upon these inter genome compatibilities. In this case, embryos from crosses among highly compatible parents may receive or be able to extract more resources from the maternal plant. Thus an increase in the average seed mass is still expected in crosses with multiple fathers if the genetic compatibility between parents mediates both the siring success and the ability of the embryo to accumulate resources. However, we do not expect paternal effect on seed mass

under this scenario, because the relative mass of the seeds sired by a particular pollen donor will depend on the compatibility with the maternal plant.

An important, but rarely appreciated, requirement for these adaptive differential maternal allocation scenarios is that the 'genetic quality' of a seed needs to explicitly manifest itself as a greater potential marginal increase in maternal fitness (recruitment probability) per capita of maternal investment (i.e. a steeper slope in the offspring fitness curve), as compared to the offspring of lower quality from less competitively successful pollen donors. An overall increase in genetic quality or fitness (e.g. recruitment probability) in seeds from highly competitive pollen irrespective of maternal investment (generating only an increase in the intercept of the offspring fitness curve) is not enough in itself to generate adaptive differential allocation by mothers in favour of the high quality offspring (Fig. 1).

Finally, it is important to keep in mind that because an extra allocation towards genetically superior embryos is expected to increase the fitness of the maternal plant, differential allocation must occur with the ìconsentî of the maternal plant, and should not lead to any increased parent offspring conflict over resources allocated to the seeds (see below). In summary, under the differential allocation hypothesis, multiple paternity within fruits should lead to an increase in average seed mass associated with biased paternity and possibly (but not necessarily) an increase in the variance in seed mass. Most importantly, under this scenario, siring success should be positively correlated with the mass of the seeds sired.

Sibling rivalry ñ Male male competition may continue over the period of seed maturation via conflict over maternal resources (Mazer, 1987). Following Hamiltonís rule on kin selection (Hamilton, 1964), sibling rivalry over maternal resources will be stronger among half sib than among full sib offspring (Trivers 1974). Assuming that paternally derived alleles in the

193 developing embryo can affect its ability to extract maternal resources, increased sibling
194 rivalry may occur between half sibs over these resources (Mock & Parker, 1997). BaÒuelos
195 and Obeso (2003) likewise suggested that sibling competition among seeds within multiply
196 sired fruits could drive the extraction of maternal resources beyond the optimum for the
197 maternal plant. This argument was based on the observation that increasing seed numbers in
198 the sweet pepper flowers (Capsicum annuum) generated an increase in fruit mass (Marcelis &
199 Hofman Eijer, 1997), therefore supporting the hypothesis that embryos were able to influence
200 resource allocation by the maternal plant. This hypothesis, however, is not necessarily
201 unconditionally predicted by theory concerning parent offspring conflict, since we might
202 expect selection on mothers to prevent such misappropriation of resources by offspring
203 against the fitness interests of the mother (Mock & Parker, 1997).
204 Indeed, the normal expectation from the parent offspring conflict theory is that
205 increased sibling competition arising from lower levels of sibling relatedness produces
206 broods with more variable offspring size and/or smaller brood sizes due to brood reduction
207 (Shaanker et al., 1988; Parker et al., 2002; Sakai, 2007). Because sibling competition does not
208 necessarily increase maternal fitness, it is unlikely that it leads to increased investment by the
209 maternal plant (Parker et al., 2002). Indeed, similar fitness is often expected for parents
210 providing an equal share of resources among offspring versus a hierarchical distribution of
211 resources among competing offspring (Mock & Parker 1997; Parker et al., 2002). Therefore,
212 parents should not differentially allocate energy between their broods according to the level
213 of sibling rivalry. Accordingly, several angiosperm features, such as maternally derived
214 integument or triploid endosperm, have been interpreted as evolved countermeasures against
215 selfish offspring (Westoby & Rice, 1982; Queller, 1983; Mazer, 1987; Friedman et al., 2008).
216 In conclusion, an increase in sibling rivalry among seeds in multiply sired fruits is
217 expected mostly to increase the variance in seed mass and possibly reduce the number of

seeds, but it is not expected to increase the average seed mass. Furthermore, a paternal effect on seed mass is expected if the ability of the embryo to compete for resources is determined by genes with additive effects.

Parent-offspring conflict and variation in mother-offspring relatedness In the previous scenarios, we assumed that pollen donors were genetically different from each other and from the maternal plants, so that seeds were equally related to the maternal plant with a coefficient of relatedness of 0.5. In self compatible species, however, relatedness between the developing seeds and the maternal plant may vary between 0.5 (outcross) and 1 (self pollination). This variation may affect the level of evolutionary conflict between the maternal plant and the developing seeds over resource allocation (i.e. parent offspring conflict, Trivers 1974). In such systems, if the embryo can influence the degree of maternal provisioning, a lower degree of relatedness between seeds and the maternal plant is expected to generate an increase in the demand of maternal resources by the embryo. This may lead to an increase in seed size beyond the maternal optimum, possibly at the expense of the seed number (de Jong et al., 2005). Consequently, any correlation between multiple paternity and the average genetic relatedness between dams and sires could result in an increase in the average seed mass of broods with multiple fathers. Such correlations could emerge, for example, from the greater success of pollen from sires with lower degree of relatedness with the maternal plant (e.g. Teixeira et al., 2009).

Predictions that follow from this parent offspring conflict hypothesis are relatively similar to those following from the differential allocation hypotheses. Indeed, in both cases, we expect multiple paternity to positively affect the average seed mass and the within fruit variance in seed mass. We also expect the siring success to be positively correlated with seed mass. However, the parent offspring conflict hypothesis also predicts that self pollination

should produce smaller seeds than cross pollination with single pollen donor, and that the number of seeds per fruit may be reduced under outcrossing.

Distinguishing between these different hypotheses requires information not only about average seed mass but also about the number of seeds per fruit, the within fruit variance in seed mass, the possible bias in siring success among males, and whether siring success and paternal effects on seed mass are correlated (Table 2). To illustrate this, we report the results of an experiment where we tested the effects of multiple paternity on seed mass in the neotropical vine Dalechampia scandens (Euphorbiaceae). Average seed mass, brood size and within brood variance in seed mass were compared between three kinds of within population crosses: (a) self pollination, (b) outcross with one pollen donor, and (c) outcross with three pollen donors. We genotyped all the parental plants and the offspring from the multiple pollen donors crosses in order to confirm the occurrence of broods with multiple fathers and to test differences in siring ability among pollen donors. We conducted the crosses among 68 plants controlling for their degree of relatedness.

Methods

Study species

Dalechampia scandens is a monoecious vine that produces blossoms containing male and female subinflorescences. These are together subtended by two showy bracts that play a role in attracting pollinators during the reproductive phase of the blossom (Fig. 2; PÈrez Barrales et al., 2013). During fruit maturation, these bracts play a role in both protecting the developing seeds and in providing energy for their maturation (Armbruster 1996; PÈlabon et al., 2015a). The female subinflorescence comprises three female flowers, which contain three ovules each. Thus, each blossom can produce a maximum of nine seeds. The male

268 subinflorescence comprises ten staminate flowers plus a gland producing a terpenoid resin
269 collected by female bees in the genera Eulaema, Eufriesea, Euglossa and Hypanthidium,
270 and/or worker Trigona (Armbruster, 1984, 1985). During the first 2 3 days after the blossom
271 has opened, only female flowers are receptive (female phase). After this period, the first
272 (terminal) male flower opens, followed by the opening of the other male flowers in
273 succession over a period of approximately one week (bisexual phase). The plant is self
274 compatible, and blossoms can self pollinate during the bisexual phase, although the distance
275 between the anthers and the stigmas (degree of herkogamy) affects the frequency of auto
276 pollination (Armbruster, 1988).
277 Individuals used in this study were the fifth greenhouse generation descended from
278 seeds collected from 75 maternal plants in a population in the state of Quintana Roo, Mexico
279 (20°13¢N, 87°26¢W). The greenhouse population was maintained by outcrossing for five
280 generations with always more than 200 individuals per generation. During these five
281 generations, the pedigree of each individual was recorded. In the current experiment we
282 included sixty eight individuals that were genetically distinct as indicated by the
283 microsatellite analysis (see below).
284
285 Experimental design
286 We hand pollinated blossoms in the female phase with either (i) pollen from a single donor
287 genetically distinct from the maternal plant (single-donor treatment), (ii) a mixture of pollen
288 obtained from three donors, genetically different from each other and from the maternal plant
289 (multiple-donors treatment), or (iii) pollen obtained from a blossom on the same maternal
290 plant (self-pollination treatment). The sixty eight plants were arranged in 17 blocks of four
291 plants each. Within each block, all plants were crossed in a full diallel design, each plant
292 being exposed to the three pollination treatments either as pollen donor or as pollen receiver

293 (Fig. 3). Crosses with multiple donors were duplicated (two blossoms from the same plant
294 received the mixture of pollen from the three other plants in the block). To avoid variation in
295 seed mass due to potential inbreeding depression, plants included in each block had low
296 levels of genetic relatedness (median inbreeding coefficient: 0.01, range 0 to 0.13; based on
297 the pedigree of the last four generations).
298 Blossoms in female phase were emasculated by removing the entire male
299 subinflorescence, and pollination was carried out by applying pollen with a toothpick to each
300 of the three stigmas. For the multiple male crosses, we first mixed equal amounts of pollen
301 from each paternal blossom (pollen taken from one freshly opened staminate flower per
302 paternal plant) on a microscope slide with a toothpick, and we applied the pollen mixture to
303 the tip of the stigma of each of the three female flowers. In the single male and self
304 pollination treatment, we followed the same procedure in order to perform crosses under
305 similar conditions. Microscope slides and toothpicks were changed between each cross. To
306 minimize the variation in seed mass due to variation in blossom seed set, we pollinated each
307 female flower with an excess of pollen in order to avoid variation in the number of seeds due
308 to pollen limitation. This procedure also promoted pollen competition. All crosses that
309 produced fewer than seven seeds were repeated and replaced to ensure that blossom seed sets
310 varied only between 7 and 9 seeds.
311 Variation in blossom size may affect the size of the seed produced (Herrera, 2009).
312 We therefore measured the diameter of the blossom peduncle as a proxy for blossom size
313 before and after pollination; the average of these two measurements was used as a covariate
314 in the analysis of seed mass. After pollination, blossoms were bagged in order to collect seeds
315 after explosive dehiscence five to six weeks after pollination. Collected seeds were weighted
316 individually on a precision balance (to the nearest 0.001g). The number of seeds produced by
317 the blossom (brood size) was also recorded.

318 In this study the effects of the treatment on the average seed mass were estimated at
319 the level of the infructescence, and not at the level of the single fruit (each infructescence
320 contains three fruits in D. scandens). However, because all fruits within each infructescence
321 were exposed to the same treatment, we expected the treatment effects to add up at the level
322 of the infructescence. Therefore, even if different treatments generated differences in average
323 seed mass only at the level of the fruit, we expected these effects to be exhibited also at the
324 infructescence level. Furthermore, it is also possible that several of the mechanisms described
325 above (e.g. sibling competition, differential allocation) also occur among fruits within
326 infructescence. This is particularly likely in D. scandens where the average seed mass is
327 affected by the size of the infructescence (see results).
328
329 Paternity analysis
330 To confirm that the multiple pollen donors treatment resulted in broods with multiple fathers
331 and to test whether paternal plants differed in their siring ability, we conducted paternity
332 analysis on one of the duplicated sets of crosses from the multiple father treatment. Seeds
333 were sown and the first true leaves were collected after ca. two weeks. When more than half
334 of the seeds from a particular cross failed to germinate, the cross was discarded, and we
335 sowed all the seeds from the other duplicate cross.
336 Paternity was inferred by first screening all individuals from the parental generation
337 for polymorphism at 70 microsatellite markers (Falahati Anbaran et al. 2013). A subset of
338 polymorphic markers was then used to genotype both parents and offspring. Genomic DNA
339 was extracted from the dried leaf tissues using an EZ 96 Plant DNA Kit (OMEGA BIO TEK,
340 Norcross, GA, USA). PCR reactions were performed using 5 l Type it Microsatellite PCR
341 Kit (Qiagen, Hilden, Germany), 1 l 10x primer mix (each primer with different
342 concentration, Table S1), 1 l genomic DNA in a total volume of 10 l. Thermal profile was

343 set at 94 ° C for 4 min for one cycle, 94 ° C for 50 s, 65 56 °C as touchdown, each step for 45
344 s, 72 ° C for 1 min for 35 cycles followed by a final extinction of 72 °C for 15 min. The sizes
345 of microsatellite fragments were determined using ABI 3130xl (Applied Biosystems, Forster
346 City, Ca, USA). Microsatellite alleles were detected by GeneMapper V. 4 (Applied
347 Biosystems).
348 Molecular analyses revealed 13 polymorphic microsatellite markers among parental
349 genotypes (Table S1). Ten of these markers were used in a single multiplex PCR to genotype
350 567 offspring produced by crosses with multiple pollen donors. We performed parentage
351 analysis to identify the most likely father using Cervus 3.0.3 (Kalinowski et al., 2007). We
352 computed the critical Delta scores from simulating the genotypes of 10000 offspring from the
353 allele frequencies in the putative fathers and used a strict (95%) confidence level for Delta
354 scores to assign the most likely father. Additionally we inspected manually the compatibility
355 of the offspring genotype to the one of the putative parent pairs. The parentage analyses were
356 conducted for each known mother and three unknown fathers separately. Because presence of
357 null alleles and genotyping errors may affect the number and size of alleles, the parental
358 individuals were re genotyped using all microsatellite loci. No genotyping error or null
359 alleles were present at the studied loci.
360
361 Statistics
362 Variation in siring ability ñ In order to test whether males differed in their siring ability when
363 pollen competition occurred, we calculated the number of seeds sired by each of the three
364 males for each blossom in the multiple donors treatment. The siring success of each male was
365 then estimated as the total number of seeds sired given that the maximum possible siring
366 success was 27 seeds (3 blossom × 9 seeds). (Remember that we estimated paternity for only
367 one of the two replicated crosses in the multiple donors treatment.) We tested whether male

368 plants differed in their siring ability using mixed effects generalized linear models with
369 Poisson error distribution, where the fatherís identity was the predictor variable and motherís
370 identity was a random factor. This analysis is based on the fact that multinomial distributions
371 can be derived from a set of independent Poisson random variables conditioned on their sum
372 being fixed, here a fixed number of seeds (McCullagh & Nelder, 1989). Two models
373 including or not father identity as predictor variable were compared using Akaike
374 Information Criteria (AIC).
375
376 Mean and variance in seed mass ñ We tested the effect of the pollination treatments on the
377 average seed mass by comparing mixed effects models on seed mass, where pollination
378 treatment was a fixed factor, peduncle diameter a covariate, and mother plant and blossom
379 nested within mother plant were random factors. Model selection was performed using AIC
380 obtained from models fitted with maximum likelihood, and parameter estimates were
381 obtained for the best model(s) fitted with restricted maximum likelihood. To test whether the
382 variance in seed mass increased with multiple paternity, we compared models where seed
383 mass was the response variable, peduncle diameter a covariate and mother plant and blossom
384 nested within mother plant were random factors, and where different random variances for
385 the different treatments were allowed versus not. This was achieved by using the varIdent
386 function in the nlme package in R (Zuur et al., 2009). These models did not include the self
387 pollination treatment, because crosses were not replicated within maternal plant in this
388 treatment. Consequently, mother and cross were confounded in this treatment and the
389 structure of the random variance was necessarily different from the two other treatments. We
390 further estimated the different components of the random variance (among plants, among
391 blossoms within plant, and within blossom) and their 95% highest posterior density intervals
392 by fitting mixed effects models with the R package MCMCglmm (Hadfield, 2010) for each

393 treatment separately. We used zero mean Gaussian distributions with very large variances
394 (10^8) as priors for the fixed effects in the Bayesian mixed models, scaled F distributions
395 where the variance/1000 were $F_{1,1}$ distributed for the variance parameters, and inverse
396 Wishart distributions for the residuals (Hadfield, 2010). These models ran for 260,000
397 iterations, with a burn in phase of 10000 and a thinning interval of 250.
398
399 Paternal effect ñ Using the subset of the data on seed mass for which the father was known,
400 and excluding the self pollination treatment, we tested for the occurrence of a paternal effect
401 on seed mass by fitting mixed effects models where seed mass was the response variable,
402 peduncle diameter a covariate, and father, mother and blossom nested within mother were
403 random factors. We obtained the different components of the random variance and their 95%
404 highest posterior density intervals using MCMCglmm (see above) for the whole subset of
405 data including treatment as fixed effect, and for the two treatments (single and multiple
406 donors) analyzed separately.
407 Finally, we tested whether the ability to sire seeds was positively correlated with the
408 mass of the seed sired by testing the correlation between the number of seeds sired by each
409 male in the multiple males treatment and the average mass of these seeds once the maternal
410 and blossom effects were accounted for. The relative seed mass for each male was estimated
411 as the average male residuals from a linear model with seed mass as response variable and
412 peduncle diameter and maternal plant identity as predictor variables. We included seeds
413 obtained from both single and multiple donors treatments.
414

415 Results

416 Variation in siring ability

417 The number of seeds produced per blossom was not affected by the treatment (model
418 including treatment AIC = 549.21; model including only an intercept AIC = 545.64), with an
419 average (±SE) of 8.86 ±0.04 seeds per blossom in the multiple donors treatment, and 8.86
420 ±0.03 and 8.82 ±0.06 seeds per blossom in the single donor and self pollination treatment,
421 respectively. In the multiple donors treatment, sires obtained an average of 2.78 ±0.15 seeds
422 per blossom, with a paternity success ranging from 0 to 9 seeds per blossom. When
423 considering the three crosses in which each pollen donor competes with two other donors, the
424 siring success varied from 1 to 20 seeds, that is, from 4% to 74% of the maximum available
425 seeds. Pollen donors differed in their ability to sire seeds, as indicated by the better fit of the
426 model including father identity as predictor variable to explain the number of seed sired
427 (model including father identity AIC = 861.4; model including only an intercept AIC =
428 875.7).
429
430 Effects of treatments on the mean and variance in seed mass
431 Individual seeds had an average mass of 38.89 ±0.26 mg, which increased with an increasing
432 peduncle diameter (β = 7.46 ±1.54 mg mm^{-1}) and tended to decrease with an increasing
433 number of seeds per fruit (β = −0.35 ±0.33 mg seed^{-1}), although this last effect was poorly
434 supported statistically (Table 3). We found no marked effect of treatment on seed mass
435 (Table 3): the average seed mass in the different treatments was 38.97 ±0.30 mg, 38.68 ±0.33
436 mg and 39.08 ±0.41 mg in the multiple donors, single donor and self pollination treatment,
437 respectively.
438 Variance in seed mass did not increase in the treatment with multiple fathers, as
439 indicated by the better fit of the model including only a single term for the random variances
440 (inclusion of different random terms for each treatment increases the AIC by 0.81). Similarly,
441 the distributions of the variance within blossom, among blossoms and among plants did not

differ among treatments (Table 4), confirming that the within brood variance in seed mass did not increase in the multiple pollen donors treatment. We noticed, however, that the phenotypic variance in seed mass tended to be less in seeds produced by self pollination (Table 4).

Paternal effect and correlation between siring success and seed mass

Overall, the paternal effect on seed mass was very limited and never supported statistically because the lower limit of the highest posterior density intervals was always very close to zero (Table 5). Nevertheless, while the paternal component of the random variance was nearly zero in the multiple donors treatment, it reached a median value of 3.8% of the total random variance in seed mass in the single pollen donor treatment (Table 5). Finally, the siring success and the relative mass of the seeds sired by a male were not correlated ($r = 0.14$; $df = 66$; $P = 0.25$).

Discussion

Studies testing for an increase in seed mass when seeds from the same fruit or infructescence are sired by multiple pollen donors have provided very limited support for this prediction (Table 1). Our own study on D. scandens conforms to this pattern, because we found no difference in mass among seeds produced by blossoms pollinated by either multiple or single pollen donors. This general lack of effect may have several causes, but often, it appears that the necessary assumptions for an increase in seed mass for multiply sired fruits are not met. In the following, focusing primarily on our own results, we try to identify critical aspects of the angiosperms reproduction that may prevent the occurrence of such an effect.

Paternal effect on seed mass, differential allocation and sibling rivalry

Differences in siring success among pollen donors observed in our study opens up the possibility for mechanisms associated with sexual selection and differential allocation to affect seed mass whenever multiple paternity occurred within fruits. Furthermore, despite the limited variation in seed mass in D. scandens (CV^a10%, Table 4) compared to a large number of species (Michaels et al., 1988; Susko & Lovett Doust, 2000; Turnbull et al., 2006), more than 67% of the variation in seed mass is expressed among blossoms within plants (Table 4). Although part of this variation is explained by differences in blossom size that can result from positional effects (e.g. Stanton, 1984; Diggle, 1995), a large part remains potentially available for paternal effects or differential maternal allocation. However, the paternal effect on seed mass estimated in this study was particularly weak and confirmed the results of a precedent study on the same population were paternal effects explained at most 4% of the seed mass variation (Pelabon et al. 2015b). Furthermore, the pollination treatment did not significantly affect seed mass indicating that no differential allocation occurred in multiply sired fruits. The complete absence of correlations between siring success and the mass of the seeds sired also suggests that mechanisms linked to sexual selection and differential allocation are unlikely to operate in our study species. The weak paternal effect on seed mass also limits the opportunity for sibling rivalry within blossoms, as confirmed by the absence of an increase in within blossom variance in seed mass in the multiple donors treatment.

When considering other species, only three of the 11 studies that tested the effects of multiple paternity on seed mass also assessed the magnitude of the paternal effect on this trait (Table 1). In each case, this paternal effect represented less than 4% of the variation in seed size or seed mass. Although limited to few species, this information suggests that the opportunity for inter sexual conflict or sibling rivalry over seed size is often limited by the weakness or absence of paternal effect on seed mass in angiosperms.

492 Paternal effects may be limited in species with high levels of self fertilization (de
493 Jong & Scott, 2007). Although self pollination occurs in natural populations of D. scandens
494 (PÈrez Barrales et al., 2013), molecular data indicate that this species also reproduce to some
495 extent by out crossing (Falahati Anbaran, Bolstad, PÈlabon, Armbruster, unpublished
496 results). Furthermore, the population on which this experiment was conducted is
497 characterized by a relatively large degree of herkogamy and a low selfing rate via autogamy
498 (Opedal, PÈlabon, Armbruster unpublished). Therefore, we infer that the level of selfing in
499 the study population is not sufficient to prevent the occurrence of paternal effects in seed
500 mass. Additionally, the paternal effect observed in our study species is of similar magnitude
501 to the paternal effects observed in natural populations of angiosperms, even in self
502 incompatible species (Marshall & Ellstrand, 1986; Marshall, 1988; Diggle et al., 2010) and in
503 species reproducing mostly via outcrossing (Fenster, 1991; Byers et al., 1998).
504 These results contrast with those obtained in Arabidopsis, where paternal effects on
505 seed mass as large as 10% have been reported (House et al., 2010; de Jong et al. 2011). These
506 effects resulting from crosses among different accessions (i.e. crosses among populations) are
507 true genetic effects, but whether or not they are comparable with any within population
508 paternal effects, that is, paternal effects normally involved in sexual selection processes,
509 remains an open question. Indeed, if seed mass is determined by genes with additive effects
510 expressed in the offspring to increase the seed mass and in the mother to maintain seed mass
511 at the optimal value for the maternal plant, we expect these conflicting effects to come to
512 some equilibrium with fixed alleles within population (de Jong et al., 2011). Because
513 different alleles may have gone to fixation in different populations, maternal countermeasures
514 may prove ineffective when exposed to novel paternally derived alleles during inter
515 population crosses. Thus, we may expect paternal effects on seed mass to be much greater
516 during inter population crosses than during within population crosses.

517 If paternal effects on seed mass are generally limited in angiosperms, as suggested by
518 empirical data, sexual selection and sibling rivalry mechanisms may not be particularly likely
519 to affect seed mass in fruits or infructescences with multiple fathers.
520
521 Genetic diversity and maternal allocation
522 An increase in the mass of seeds sired by multiple fathers could also result from a differential
523 allocation of resources by the maternal plants to infructescences containing seeds with higher
524 reproductive potential. Such an increase in the reproductive potential of broods sired by
525 multiple pollen donors is expected if the genetic diversity of the seeds increases the
526 effectiveness of resource partitioning (Barton & Post, 1986), or the probability for a seed to
527 be fit in one of a range of unpredictable environments (diversification bet hedging; Yasui,
528 1998, 2001). While a study on the annual grass Triplasis purpurea lends support to the
529 resource partitioning hypothesis (Cheplick & Kane, 2004), many other studies have failed to
530 find a decrease in competition during growth with decreasing relatedness among offspring
531 (Willson et al., 1987; Kelley, 1989; Cheplick & Salvadori, 1991; Argyres & Schmitt, 1992;
532 Karron & Marshall, 1993; Donohue, 2003, Milla et al., 2012). Furthermore, evolution of
533 diversification bet hedging in plants has been extensively studied with respect to seed
534 dormancy (Simons & Johnston, 2006; Venable, 2007; see Childs et al., 2010 and Simons,
535 2011 for reviews), but genetic variation among seeds is generally not invoked as source of
536 phenotypic variation in this trait.
537 Another issue regarding the link between the genetic diversity of a brood and its
538 reproductive potential is that allocating extra resources to genetically diverse broods is only
539 expected to have an effect on the maternal fitness if mothers are limited in the number of
540 broods they can produce. For species producing a large number of flowers and fruits, having
541 many flowers pollinated by single but different pollen donors (among flower bet hedging) is

542 expected to lead to the same outcome as having single flowers pollinated by multiple pollen
543 donors (within flower bet hedging). Therefore, there seems to be little reason to expect
544 mothers to allocate extra resources to flowers pollinated by multiple pollen donors in plant
545 species that produce a large number of flowers during their lifetime, such as D. scandens and
546 many of the other species included in our review.
547
548 Parent-offspring conflict and the relatedness between mother and offspring
549 If embryos are able to influence the amount of energy extracted from the mother, we expect
550 paternally derived alleles to promote the production of larger seeds compared with maternally
551 derived alleles (Haig & Westoby, 1991). Consequently, in species with mixed mating system,
552 seeds produced via outcrossing are expected to be larger than those produced by self
553 fertilization (de Jong et al., 2005). Furthermore, if multiply sired broods are generated by
554 xenogamy on average, while single father broods result mostly from selfing, we may expect
555 seeds from multiply sired broods to be larger than those from singly sired broods.
556 Unfortunately, this prediction is similar to the one following from the effects of inbreeding
557 depression, where we expect inbred seeds to be smaller than outbred ones. Several studies
558 have reported evidence for inbreeding depression on seed mass (Fenster, 1991; Wolfe, 1993,
559 1995; Lankinen & Armbruster, 2007; Shleuning et al., 2011). Although this pattern is not
560 general (Sork & Schemske, 1992; Carr & Dudash, 1995; Byers, 1998), it can still obscure the
561 results of studies where an increase in the number of pollen donors coincides with a decrease
562 in relatedness among parents (e.g. Schemske & Pautler, 1984).
563 In our experiment, seeds produced by self pollination were slightly larger than
564 outcrossed ones. This result rules out the hypothesis concerning inbreeding depression in
565 seed size in the study population. We note that although the use of a large amount of pollen
566 on the tip of the stigma during hand pollination could have decreased the magnitude of

potential inbreeding depression (Armbruster & Rogers, 2004), the absence of inbreeding depression in the study population has been confirmed by other studies. Bengtson (2006) showed that self pollination did not negatively affect germination or survival, and no difference in blossom morphology was found between individuals produced by self or outcross pollination in the same population (Hansen et al., 2003; PÈlabon et al., 2004). This result also suggests that inter sexual conflict over seed mass is not important in our population of D. scandens, because seeds resulting from self pollination were not smaller than those produced by outcrossing.

Conclusion

A number of verbal and theoretical models have been put forward suggesting that multiple paternity among seeds within fruit may positively affect seed mass. We have shown here that this is rarely the case. When taking a closer look at some of the critical assumptions upon which these models are based, we see that in most cases, these assumptions are not fulfilled in a large number of angiosperms species. For example, the paternal effects on seed mass necessary for the occurrence of sibling competition or other mechanisms related to sexual selection are generally weak or even absent. Similarly, the higher quality of genetically diverse seeds within a fruit is rarely demonstrated, therefore limiting the potential for an increase in fitness for a maternal plant allocating more resources to these broods. Therefore, whilst we would acknowledge that within fruit multiple paternity has the theoretical potential to influence seed mass, we would also caution that this may be under particularly restrictive conditions that rarely hold in angiosperms.

These results also suggest that the fitness advantages of multiple paternity within fruit or infructescence might be limited for the maternal plants. If true, this would further suggest that multiple paternity occurs mostly as a byproduct of male male competition. Still, maternal

592 plants may benefit from pollen competition if this latter improves the genetic quality
593 (additive or non additive) of the offspring produced. However, as described in Figure 1a,
594 such an increase in the quality of the seeds produced does not necessarily requires the
595 maternal plant to allocate differentially her resources to those seeds, meaning that an increase
596 in maternal fitness via pollen competition is not expected to affect the maternal allocation to
597 the seeds.
598

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603

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884 Table 1: Studies that have tested the effects of multiple paternity on average seed mass. For each study, we report the effect of multiple paternity
885 on the average seed mass, variance in seed mass, whether or not authors found a trade off between size and number of seeds within
886 infructescence, and whether or not paternity was biased. We also report the within infructescence CV in seed mass and the possible paternal
887 effect as the percentage of variance explained by the father. ì ì indicates that data were not available because they were not estimated in the
888 original study.
889

Species	Number of donors	Effect on average seed mass	Effect on variance	Trade off size/number	Bias in Paternity[a]	CV seed size (%)[b]	Paternal Effect (%)[c]	Reference
Campsis radicans	5	No	positive			22 - 33		Bertin 1986
Chamaecrista fasciculata	5	No			No	14	No	Sork & Schemske 1992
Costus allenii	5	No (#)				13		Schemske & Pautler 1984
Crinum erubescens	10	negative	Inconclusive (*)			50 - 70		Manasse & Stanton 1991
Dalechampia scandens	3	No	No	No	Yes	10	0.1 ñ 3.8	This study
Pachycereus schottii	4	No	No	No		24		Holland et al. 2009
Raphanus raphanistrum	3	No				-		Snow 1990
Raphanus sativus	3	No			Inconclusive (•)	-	1.7 ñ 3.6	Marshall & Ellstrand 1986
Raphanus sativus	3	No ($)	No	No		16 - 20	< 1	Marshall 1988
Rhamnus alpinus	3	positive				-		Banuelos & Obeso 2003
Silene latifolia	2	No			Yes (£)	-		Burkhardt et al. 2009
Vaccinium corymbosum	3	No	No			-		Vander Kloet & Tosh 1984

890 a Whether or not siring success differs from the prediction of a random success
891 b Standard deviation in seed mass at the fruit or infructescence level divided by the average seed mass
892 c Percentage of variance in seed mass explained by the identity of the father
893 * The SD seems to decrease with multiple pollen donors, but the effect is reversed when correcting for difference in mean size (CV).
894 # There is a tendency for seed mass to increase with the number of pollen donors, but this effect is confounded with the physical and most likely genetic distance from the
895 maternal plant.
896 £ The difference in pollination success was generated by non simultaneous application of the pollen for the two donors.
897 $ Although the total weight of seeds increases in multiply sired fruits, the mean seed weight is not affected (Table 2 in Marshall 1988)
898 • Some variation in the siring success seems to be due to incompatibility among plants.

Table 2: Summary of the different hypotheses suggesting an increase in seed mass in crosses with multiple fathers. Predictions regarding the occurrence of paternal effects on seed mass, biased paternity, correlations between siring success and paternal effect, and the effect on within fruit variance in seed mass are also reported (see text for more details).

Hypothesis	Mechanisms for seed mass increase	Paternal effect on seed mass	Bias in paternity	Correlation seed mass siring success	Within brood variance	Brood reduction
Genetic diversity						
Diversification bet hedging						
Genetically diverse broods have higher reproductive potential in varying environments	Extra allocation to the whole brood	No	No (females should favor most diverse brood)	No	Constant	No
Resource partitioning						
Genetically diverse broods have higher reproductive potential via a decrease in competition after dispersal	Extra allocation to the whole brood	No	No (females should favor most diverse brood)	No	Constant	No
Differential allocation						
Siring ability positively correlates with genetic quality of the offspring	Resource allocation biased towards high quality seeds	Yes / No#	Yes	Yes	Constant or weakly increase	No
Siring ability positively correlates with the ability to extract maternal resources	Resource allocation biased towards high quality seeds	Yes / No#	Yes	Yes	Constant or decrease	No
Sibling rivalry						
Stronger competition among half sibs	increased demand on the maternal plant	Yes	No	No	Increase	Yes
Parent-offspring conflict and variation in mother-offspring relatedness						
Less related offspring extract more resources from the mother	Resource allocation biased towards less related seeds	No	Yes	Yes	Constant or increase	Yes

depends upon whether the genes responsible for the different quality among seeds have additive or non additive effects. See text for details.

905 Table 3: Model selection for the effect of pollination treatment (treat.), peduncle diameter
906 (ped.) and seed set on seed mass. AIC values and AIC weights are obtained for models fitted
907 with maximum likelihood. In all models, mother plant identity and blossom identity nested
908 within mother plant were used as random factors. Interactions between predictor variables are
909 noted as ×; the last model only includes an intercept (~1).
910

Model	AIC	AIC	AIC weight
Seed mass ~ ped. + treat. + seed set + ped.×treat.	14699.97	7.00	0.02
Seed mass ~ ped. + treat.+ seed set	14696.76	3.80	0.08
Seed mass ~ ped. + seed set	14693.88	0.91	0.35
Seed mass ~ ped.	14692.96	0	0.55
Seed mass ~ 1	14713.74	20.78	0

911
912

Table 4: Variance components for the seed mass in the different treatments. These components of the random variance were estimated using the complete data set (i.e. including seeds in the multiple donors treatment with unknown father).

Level of variation	Multiple donors	Single donors	Self
Among plants	2.75 (3.80×10^{-4}; 5.61) 17.05% (0.07; 37.02)	2.02 (5.15×10^{-4}; 4.14) 14.31% (0.88; 29.54)	10.54 (7.09; 14.28) 83.13% (77.98; 87.92)
Among blossoms /within plant	10.69 (6.88; 14.32) 67.07% (47.35; 85.24)	9.49 (7.45; 12.12) 68.60% (54.64; 81.71)	
Within blossom	2.50 (2.29; 2.70) 15.88% (12.56; 19.25)	2.35 (2.20; 2.50) 17.10% (13.98; 20.19)	2.08 (1.87; 2.36) 16.87% (12.08; 22.02)
Total variance (95% CI) [£]	15.20 (13.88; 16.56)	13.37 (12.44; 14.36)	11.95 (10.72; 13.25)
CV[#]	10.31% (9.84; 10.80)	9.80% (9.44; 10.18)	8.92% (8.39; 9.45)

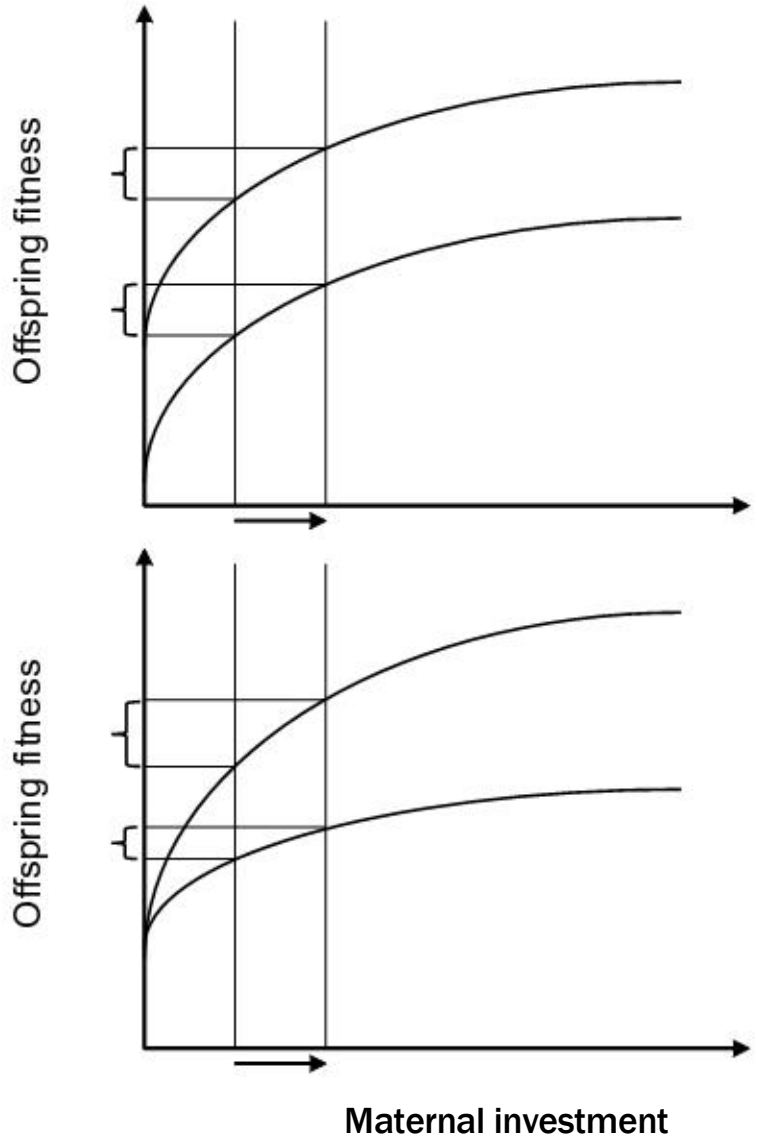
[£] Variance and 95% highest posterior density intervals obtained from mixed effects models with MCMCglmm (see Methods).

[#] CV and 95% Confidence Interval obtained by bootstrapping on the raw data on seed mass.

Table 5: Variance components for the seed mass including the effect of the pollen donor. The effect is given by the percentage of variance in seed mass explained by the pollen donor in a variance components analysis. The analysis was run for both treatments separately (Multiple and Single pollen donor) and for the whole data set (excluding self cross). The total variance for the multiple donors treatment is not the same as in table 3 because it includes here only offspring with known father.

Level of variation	Multiple donors	Single donors	Global
Among pollen donors	0.022 (1.80×10^{-10}; 0.086) 0.13% (0.00; 0.62)	0.535 (4.24×10^{-6}; 1.725) 3.77% (0.01; 13.62)	0.025 (2.27×10^{-9}; 0.099) 0.18% (1.36×10^{-4}; 0.84)
Among mother plants	5.28 (3.39×10^{-6}; 13.33) 29.94% (0.13; 77.28)	2.14 (7.30×10^{-3}; 4.13) 15.09% (1.60; 29.86)	3.49 (1.78; 6.04) 26.28% (14.84; 38.09)
Among blossoms /within plant	9.85 (0.80; 17.41) 55.86% (9.12; 87.20)	8.98 (6.69; 11.41) 64.19% (49.03; 78.85)	8.37 (6.75; 10.09) 57.28% (47.12; 68.23)
Within blossom	2.42 (2.12; 2.75) 14.06% (10.25; 18.72)	2.36 (2.19; 2.51) 16.94% (13.98; 19.98)	2.37 (2.23; 2.51) 16.26% (13.44; 18.89)

930 Figure legend
931
932 Fig. 1: Figure illustrating the effect of differential allocation of maternal investment in seeds
933 of varying quality on the offspring fitness (solid line: good quality; dash line: poor quality).
934 In the upper panel, an increase in maternal investment (represented by the arrow on the x
935 axis) does not provide a greater increase in the fitness of good versus bad quality offspring. In
936 the lower panel, however, an increase in maternal investment provides a larger increase in
937 fitness in the good quality as compared with the bad quality offspring.
938
939 Fig. 2: Blossom of the Dalechampia scandens. Arrows designate the male and female
940 flowers. The ìfemaleî arrow points towards the styles of the female flowers while the two
941 ìmaleî arrows point toward one open and one closed male flower. (Photo C. Pelabon)
942
943 Fig. 3: Schematic representation of the crossing design in each of the 17 blocks. The four
944 individuals in the blocks are represented by the four letters A, B, C and D. Each individual
945 was crossed as a mother with each of the other individuals (single pollen donor cross : ×),
946 with her self (self cross: S) and with a mixture of the pollen from the three other males
947 (multiple pollen donors; last column).


Offspring fitness
Offspring fitness
Maternal investment

Pélabon et al. Fig. 2

	Single pollen donor				Multiple pollen donors
Dams	A	B	C	D	
A	S	×	×	×	BCD
B	×	S	×	×	ACD
C	×	×	S	×	ABD
D	×	×	×	S	ABC

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Supplementary material

Table S1 - Characteristics of microsatellite loci in parental individuals of Dalechampia scandens. Ho and He are the observed and expected heterozygosity in the parental population.

Locus	Dye	Concentration (μM)	Number of alleles	H_O	H_E	Allele sizes (bp)
CCdi29	HEX	1.5	2	0.027	0.027	73,75
CCdi50	6-FAM	1.5	2	0.375	0.398	291,301
CCtri8	PET	1.5	2	0.384	0.424	195, 198
CCtri17	6-FAM	1.5	2	0.149	0.139	220,223
CCtri21	6-FAM	1.5	2	0.081	0.078	138,157
Edi6	6-FAM	1.5	2	0.135	0.127	179, 183
Edi13	NED	1	3	0.568	0.561	114,116,119
Edi15	NED	1	2	0.216	0.194	146, 148
Edi30	HEX	1.5	2	0.378	0.325	169,187
Edi17	6-FAM	1.5	2	0.356	0.374	298,305
Etri2	6-FAM	1.5	2	0.507	0.497	270,276
Etri9	6-FAM	1.5	2	0.493	0.444	228,231
Etri15	NED	1.5	2	0.473	0.488	117,190
Mean			2.08	0.32	0.31	
SE			0.08	0.05	0.05	

CCdi29, Edi17 and Etri15 were not used for the paternity analysis, because they shared alleles to markers with similar allele sizes.